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A Rapid Semiautomatical Enzyme Linked Immunoassay Identifying Intercellular Adhesion Molecule-1 (ICAM-1) on the Alveolar Macrophage Surface

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Summary: Intercellular adhesion molecule-1 (ICAM-1), a member of the immunoglobulin gene superfamily, is a cytokine-inducible adhesion molecule, which plays a central role in leukocyte migration into sites of acute or chronic inflammation. In this article we describe a sandwich immunoenzymometric method which allows rapid, semiquantitative (in "enzyme immunoassay units", EU) identification of ICAM-1 on the surface of alveolar macrophages. We evaluated this method in two groups of patients with pulmonary sarcoidosis (n = 12) or bacterial pneumonia (n = 11) and a group of healthy volunteers (n = 6), comparing the results with those obtained by immunocytochemical staining. ICAM-1 expression on the sarcoid alveolar macrophages surface was significantly elevated, as compared with control alveolar macrophages (0.76 EU ± 0.27 vs. 0.44 EU ± 0.12, $p < 0.01$). ICAM-1 expression on the surface of alveolar macrophages from patients with pneumonia was not elevated (0.48 EU ± 0.35). Stimulation with tumour necrosis factor-α (TNF-α) or interferon-γ (100 kU/l) led to a significant induction of ICAM-1 on the surface of control alveolar macrophages (0.76 EU ± 0.18, $p < 0.005$ for TNF-α, 0.64 EU ± 0.10, $p < 0.005$ for interferon-γ), whereas alveolar macrophages from both patient groups did not respond to cytokines even at high dosages. ICAM-1 expression on the surface of alveolar macrophages from patients with sarcoidosis correlated with the spontaneous release of TNF-α by macrophages ($R = 0.77$, $p < 0.05$). To summarize, the evaluation of ICAM-1 levels on the surface of cells harvested from the pulmonary compartment is a useful tool for interpreting the mechanisms leading to leukocyte accumulation and activation in inflammatory disorders of the lung.

Introduction

Intercellular adhesion molecule-1 (ICAM-1) is a cytokine-inducible adhesion molecule expressed on a wide variety of cells at sites of inflammation (1, 2). It is the principal receptor for the integrin lymphocyte function associated antigen-1 (3) and mediates a number of adhesion events among leukocytes and between leukocytes and other cells types (1, 4). ICAM-1[1]) plays a major role in controlling leukocyte trafficking into inflamed organs (5, 6). Structurally, ICAM-1 is a member of the immunoglobulin supergene family with 5 immunoglobulin-like domains (7). Considering current knowledge on the function of ICAM-1 during inflammation, studies regarding the possible role of ICAM-1 expression on the surface of alveolar macrophages in the pathogenesis of inflammatory disorders of the lung are remarkably rare (8–10). We therefore developed a rapid and reliable method for identification of ICAM-1 levels on the surface of alveolar macrophages harvested by bronchoalveolar lavage. Based on cell-physiological ELISA methods (11), we describe in this report an immunoenzymometric assay technique, which allows the semi-automatic, semiquantitative and reproducible detection of ICAM-1 on

[1]) Abbreviations: ICAM-1 (Intercellular Adhesion Molecule-1, LFA-1 (lymphocyte function associated antigen-1), EU (Enzyme immunoassay-unit), TNF-α (tumour necrosis factor-α), IFN-gamma (interferon-γ).

the surface of alveolar macrophages. We evaluated this method in two groups of patients with pulmonary sarcoidosis or bacterial pneumonia, comparing the results with those obtained from alveolar macrophages of healthy volunteers. We examined both basal and stimulated ICAM-1 expression following 16 hours of incubation with interferon-γ or TNF-α. ICAM-1 expression in the sarcoidosis group was compared with TNF-α concentration in the supernatants of alveolar macrophages as an established characteristic of macrophage activity (10). Patients suffering from pulmonary sarcoidosis were chosen because this disease provides an example of a strongly compartmentalized inflammatory disorder with accumulation of highly activated macrophages and lymphocytes in the lung (13–20). Patients with bacterial pneumonia were chosen because this disease, in contrast, is characterized by neutrophil accumulation in the pulmonary compartment.

Materials and Methods

Reagents

Human recombinant tumour necrosis factor-α and human recombinant interferon-γ were obtained from Serva, Heidelberg, Germany. Stock solutions of cytokines were stored aliquoted at −80 °C. The freeze-dried monoclonal mouse antibody against human ICAM-1 (clone 84H10) was purchased from Dianova, Hamburg, Germany. The antibody was reconstituted with phosphate-buffered saline (0.1 mol/l, pH 7.2) containing 1 g/l bovine serum albumin (Serva, Heidelberg, Germany) and sodium azide (1 g/l) to a final antibody concentration of 0.1 g/l. This stock solution could be stored at 4 °C for up to three months. Monoclonal antibodies against CD3, CD4, and CD8 were purchased from Dianova, Hamburg, Germany and Ortho Diagnostics, Beerse, Belgium, respectively. Peroxidase conjugated goat anti-mouse antibody was obtained from Sigma, Deisenhofen, Germany. Appropriate stock solutions of these antibodies in 1 g/l bovine serum albumin were stored at 4 °C and dissolved in phosphate-buffered saline (0.1 mol/l, pH 7.2) for use in the experiments. The neutralizing anti-TNF-α antibody was obtained through H. Biermann, Bad Nauheim, Germany from British Biotechnology and was kept aliquoted at −20 °C. 2,2'-Azino-bis(3-ethylbenzthiazoline-6-sulphonic acid) diammonium salt (ABTS) was purchased from Sigma. Reagents for immunocytochemical staining were from Dako, Hamburg, Germany. All cell culture media and supplements were obtained from Gibco, Eggenstein, Germany.

Study population

The study population consisted of 12 consecutive, untreated patients with active biopsy-proven sarcoidosis of the lung (radiological type I: n = 9, type II: n = 3) and 11 untreated patients with clinical and radiological signs of bacterial pneumonia. As controls, six healthy volunteers were studied. The three groups were comparable with respect to smoking habits, which have been shown to influence expression of adhesion molecules (21).

Ethical committee approval and individual informed consent were obtained.

Tab. 1 Age, sex and lavage cell characteristics of patients and healthy volunteers.

	Pneumonia n = 11	Sarcoidosis n = 12	Control n = 6
Age (a)	49 ± 23	36.7 ± 14	27 ± 4
Sex			
– male	8	4	6
– female	3	8	0
Bronchoalveolar lavage			
Total cell count (10^6)	27 ± 23	24 ± 20	11 ± 6
Alveolar macrophages (%)	77 ± 15	65 ± 19	90 ± 7
Lymphocytes (%)	4 ± 4	32 ± 19	8 ± 5
Granulocytes (%)	16 ± 10	1 ± 1	1 ± 1
Eosinophils (%)	1 ± 1	1 ± 1	
CD4+ (%)		82 ± 7	
CD4+/CD8+		8.8 ± 4.6	

Clinical data and lavage cell characteristics are summarized in table 1.

Bronchoalveolar lavage and cell isolation

Bronchoalveolar lavage was performed using a flexible fiberscope in the right middle lobe under standard conditions (22). Lavage volume was 200 ml with a mean recovery of 84%, and was not statistically different in patients and controls. The first 20 ml aliquot of the lavage, which is known to be contaminated with bronchial secretions (23), was discarded and remaining portions were pooled. After evaluation of total cell count on a haemocytometer (Neubauer), lavage cell differentials were determined by *Wright-Giemsa* staining of cytocentrifuge preparations (Cytospin II, Shandon). Cell viability was assessed using trypan blue dye exclusion (88.7% ± 6.6 for all cases). For microscopical differentiation of lymphocyte subsets (CD3, CD4, CD8), fluorescein-conjugated monoclonal antibodies were used. Lavage cells were washed twice using phosphate-buffered saline and resuspended at a viable alveolar macrophages concentration of 10^9/l in M199 supplemented with foetal calf serum (50 ml/l, Gibco), *L*-glutamine (2 mmol/l, Gibco) and penicillin/streptomycin (1 g/l, Gibco).

ICAM-1 expression on the surface of alveolar macrophages

ICAM-1 expression on the surface of alveolar macrophages was determined using an immunoenzymometric assay technique. Mononuclear lavage cells containing viable alveolar macrophages at a density of 10^9/l were seeded into 96-well flat bottom microtitre plates (100 µl/well) for three hours (37 °C, 5% CO_2) to allow the macrophages to adhere. We chose this cell concentrations from the linear portion of the cell number/absorbance curve. Non-adherent cells were removed by gently washing three times with warm cell culture medium. Serum-supplemented M199 (100 µl/well) with or without 100 kU/l interferon-γ or TNF-α was added. Incubation was continued for 16 hours at 37 °C in a totally humidified atmosphere containing 5% CO_2. At the end of the incubation time cells were washed twice and fixed with 10 g/l paraformaldehyde (1 h, 21 °C). After washing the fixed cells three times with phosphate-buffered saline (0.1 mol/l, pH 7.2) free binding sites were blocked by adding a 20 g/l solution of bovine serum albumin diluted in phosphate-buffered saline, 0.1 mol/l for 1 hour at 37 °C. The blocking solution was removed by inversion of the microtitre plate and gently tap-

ping it on a soft paper tissue. A total of 100 µl of the monoclonal mouse anti-ICAM-1 antibody in an antigen-saturating concentration (1 : 1000, containing 1 g/l bovine serum albumin) was added to each well for two hours at 37 °C. At the end of the two hours, plates were washed three times and 100 µl of the developing antibody was added (peroxidase conjugated goat anti-mouse antibody, 1 : 1000, containing 1 g/l bovine serum albumin). After incubating the plates for 1 hour at 37 °C, the enzyme conjugate was removed and plates were washed four times with phosphate-buffered saline. Finally ABTS (1 g/l) and hydrogen peroxide (2.8 µl 300 g/kg H_2O_2 per 10 ml) in 0.1 mol/l sodium acetate, (pH 4.2) was added. The colour was allowed to develop for 120 minutes and plates were read with a microplate reader (Behring EL 311) at 405 nm. Appropriate controls were included in each assay. These include omission of the primary antibody and/or the developing antibody and/or the substrate or the cells. Non-specific binding of the primary monoclonal mouse antibody to alveolar macrophages was quantified using mouse IgG_1 instead of the anti-ICAM-1 antibody. Results are expressed as enzymeimmunoassay units (EU) from quadruplicate wells calculated as the difference of absorbances ($\Delta A_{405\,nm}$) measured in the wells with added anti-ICAM-1 antibody and in the control wells with added mouse IgG_1. One EU is defined as $A_{405\,nm}$ = $A_{405\,nm}$ anti-ICAM-1 antibody − $A_{405\,nm}$ mouse IgG_1; intraassay variation 9.7%. The number of adherent cells at the end of each experiment was not different in patient samples and controls as quantified by crystal violet staining. Cell viability after 16 hours of stimulation was determined randomly in separate experiments using a colorimetric assay according to *Mosmann* (24).

Immunocytochemical staining

For immunocytochemical staining of ICAM-1 cytocentrifuge preparations of the lavage cells were dried at room temperature, wrapped in aluminium foil and stored at −20 °C until use. Immunocytochemical staining was performed using the alkaline phosphatase monoclonal mouse anti-alkaline phosphatase complex as described (25). Briefly, frozen slides were warmed to room temperature before unwrapping. After fixing the cells for 2 min with pure acetone and washing with Tris-buffered saline (pH 7.6), slides were incubated for 30 min at 21 °C with 200 µl of the monoclonal anti-ICAM-1 antibody (1 : 25) in a humidified chamber.

The primary antibody was removed and slides were washed twice before adding 200 µl of a rabbit anti-mouse antibody for 30 min.

After two washings with Tris-buffered saline slides were incubated with 200 µl of a 1 : 50 dilution of the alkaline phosphatase monoclonal mouse anti-alkaline phosphatase complex.

The alkaline phosphatase product was visualized using a naphthol AS-MX phosphate fast red solution (Sigma, Deisenhofen, Germany), containing 1 mol/l levamisole to inhibit endogenous alkaline phosphatase. Finally, slides were counterstained with haemalaun (20 min) and blued under running tap water (10 min).

Tumour necrosis factor-α bioassay

For determination of TNF-α, alveolar macrophages from patients with sarcoidosis and healthy volunteers were cultured in 96-well microtitre plates as described above. After removing non-adherent cells 100 µl fresh M199 supplemented with 50 ml/l foetal calf serum was added to each well. Supernatants were removed from at least 6 wells after 60 min (37 °C). Samples from triplicate wells were pooled into one tube and immediately frozen at −20 °C. TNF-α was measured using a L929 fibroblast lytic assay as previously described (26). Briefly, L929 cells (60 000/well) were cultured in 96-well flat bottom microtitre plates (Nunc) containing threefold serial dilutions of conditioned supernatant of sarcoid alveolar macrophages, in the presence of actinomycin D (1 mg/l, total assay volume: 200 µl/well). After 20 hours of incubation, the remaining L929 cells were stained with crystal violet. The TNF-α concentration of the triplicate samples was quantified by comparing the results with the linear portion of a standard curve, obtained with human recombinant TNF-α (6.6×10^6 kU/g). The specificity of this bioassay was tested by neutralizing peak samples with a goat anti-human-TNF-α antibody (0.1−100 mg/l).

Statistics

Non-parametric statistics were used throughout the study. The *Wilcoxon* signed rank test was used for paired samples and the *Mann-Whitney* U-test for independent samples. Correlations were made with the *Spearman* rank correlation. The median was used as a marker of central tendency. Differences were considered statistically significant per $p < 0.05$ (27).

Results

Total and differential cell count

Table 1 shows the lavage cell characteristics. As expected, the total number of recovered bronchoalveolar lavage cells was increased in both patient groups. The percentage of lymphocytes in the sarcoidosis group was significantly increased, compared with healthy controls ($p < 0.005$). In patients with pneumonia, neutrophils but not lymphocytes were significantly increased in bronchoalveolar lavage.

Expression of ICAM-1 on alveolar macrophages

Using the immunoenzymometric assay, ICAM-1 expression on the surface of alveolar macrophages was de-

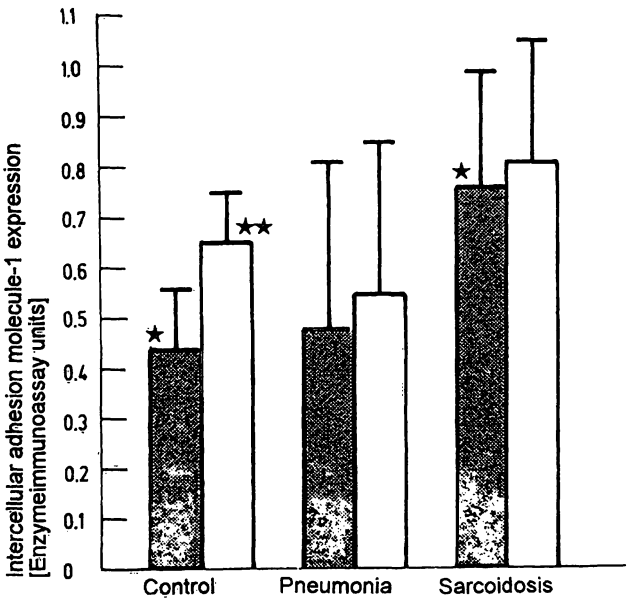

Fig. 1 Basal (dark columns) and interferon-γ induced (100 kU/l, white columns) ICAM-1 expression on the surface of alveolar macrophages from patients and controls. Mean ± SD.
* = $p < 0.01$ versus basal expression on the surface of control alveolar macrophages.
** = $p < 0.05$ versus basal expression on the surface of control alveolar macrophages.
Control (n = 6),
Pneumonia (n = 11),
Sarcoidosis (n = 12).

tected in all cases (fig. 1). Specific antibody binding was determined for each patient/control by subtracting non-specific binding of mouse IgG_1 to alveolar macrophages (fig. 2). In all cases absorbance due to non-specific binding did not exceed 10% of maximum absorbance obtained after addition of the specific anti-ICAM-1 antibody. We found significantly elevated ICAM-1 expression on the surface of sarcoid alveolar macrophages, compared with those of healthy controls (0.76 EU $\pm$ 0.27 vs. 0.44 EU $\pm$ 0.12, $p < 0.01$). In contrast, ICAM-1 expression on the surface of alveolar macrophages from patients with pneumonia was not elevated (0.48 EU $\pm$ 0.35, p = not significant).

ICAM-1 expression on the surface of alveolar macrophages from healthy volunteers was augmented after stimulation with interferon-γ or TNF-α (fig. 1). In three controls, a dose dependent increase of ICAM-1 expression was demonstrated (fig. 3). However patients' alveolar macrophages did not respond to cytokines even at high doses (500 kU/l interferon-γ or 500 kU/l TNF-α, data not shown).

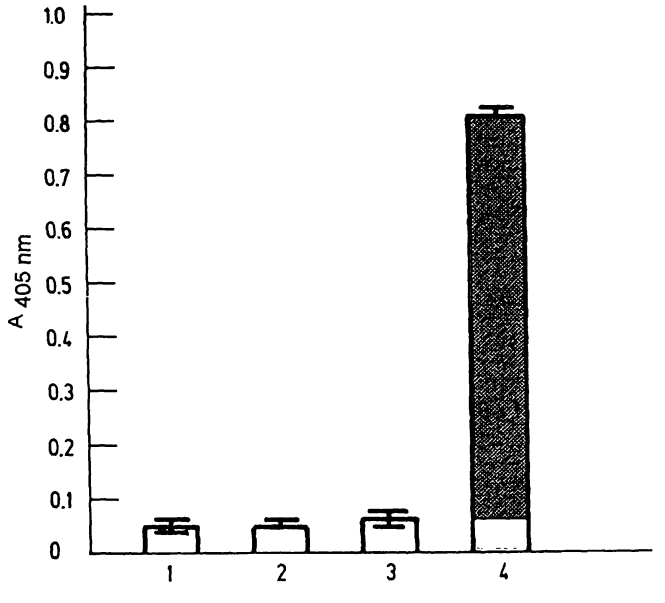

Fig. 2 Assay results given as $A_{405\,nm}$ for the expression of ICAM-1 on the surface of alveolar macrophages.
ELISA tests were performed as follows:

No.	Specific monoclonal primary-anti-ICAM-1 antibody	Mouse IgG_1	Enzyme-linked second antibody	Substrate
1	−	−	+	+
2	+	−	−	+
3	−	+	+	+
4	+	−	+	+

The black part of column 4 corresponds to the expression of the ICAM-1 on the cell surface.

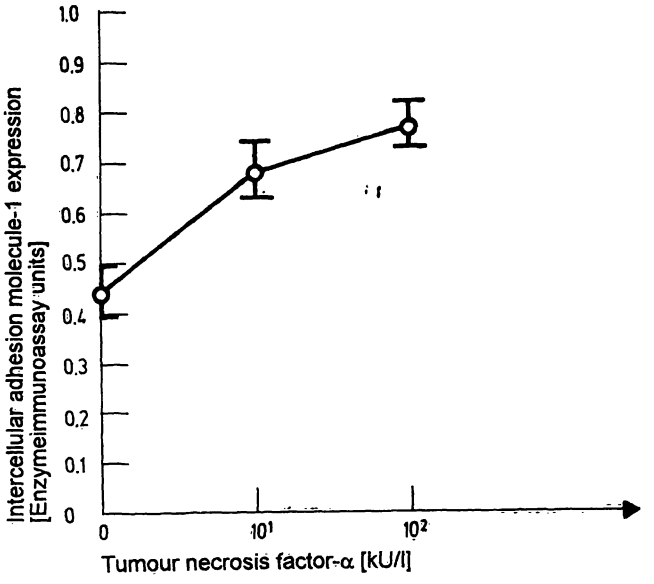

Fig. 3 TNF-α induced ICAM-1 expression on the surface of alveolar macrophages from healthy volunteers. Mean ± SD from 3 cases.

Immunocytochemical staining on cytocentrifuge preparations correlated with the results obtained with the immunoenzymometric assay method. In pulmonary sarcoidosis the number of visibly stained cells was significantly elevated when compared with healthy volunteers (78% ± 11 vs. 40% ± 8 positive cells, $p < 0.05$). Furthermore, we observed that staining of patients' alveolar macrophages resulted in more intense colouring of the cells than immunocytochemical staining of control alveolar macrophages (fig. 4).

Tumour necrosis factor-α bioassay

TNF-α was determined in the supernatants of alveolar macrophages. All sarcoid alveolar macrophages spontaneously released TNF-α, whereas only low TNF-α concentration could be detected in the supernatants of controls. TNF-α concentrations in patients ranged from 5−1375 kU/l reflecting different stages of activity of the disease. TNF-α concentration in the supernatants of sarcoid alveolar macrophages correlated with the ICAM-1 surface expression of these cells ($r = 0.77$, $p < 0.05$; 10). TNF-α was not determined in bacterial pneumonia.

Discussion

We describe a method for the rapid, semi-automatic identification of ICAM-1 on the surface of alveolar macrophages. Our results correlate with those obtained by immunocytochemical staining, a technique which has been used previously to estimate surface expression of adhesion molecules on alveolar macrophages (8, 9). We

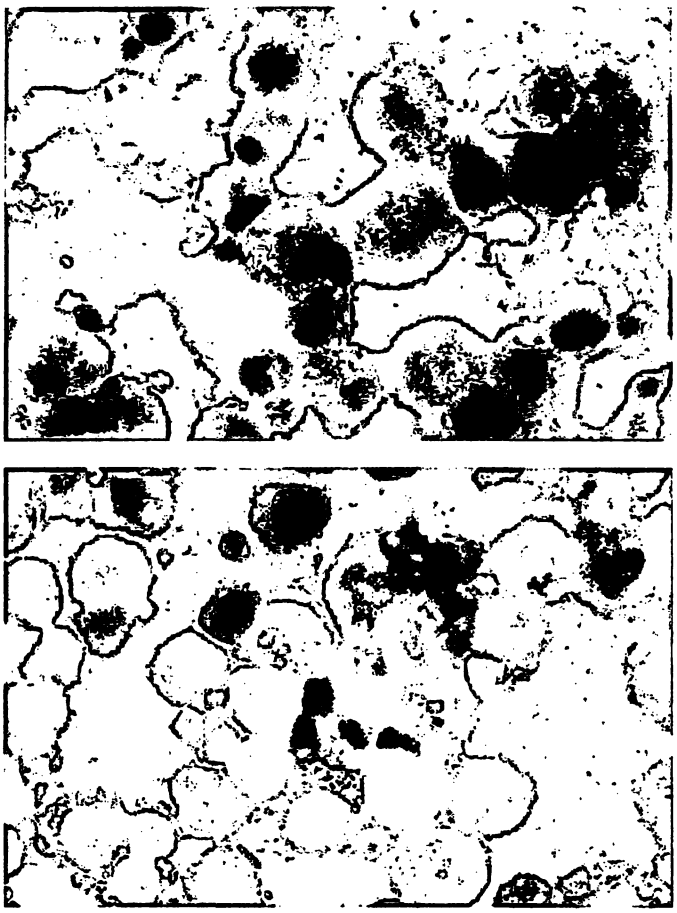

Fig. 4 Immunocytochemical staining of cytospin preparations of sarcoid alveolar macrophages (upper panel) and control alveolar macrophages (lower panel) using alkaline phosphatase monoclonal mouse anti-alkaline phosphatase complex (magnification: 400×).

did not directly compare our ELISA with flow cytometric methods, which can also be used for detection of cell surface markers. Flow cytometry is widely established for lymphocyte typing and provides the advantage of testing more than one antibody on identical cells. However alveolar macrophages show high background fluorescence, which interferes with the specific signal, especially when low receptor expression is expected (28). In this respect, we found the immunoenzymometric assay to be very precise, allowing the quantification of small differences in ICAM-1 expression that are difficult to assess by microscopic analysis. The use of a microplate reader for measuring the indicator enzyme activity at the end of the experiment is not only convenient but leads to more objective quantitative results than those obtained by microscopic evaluation of cell stains. In addition, large numbers of cells can be analysed in one experiment (e. g. investigation of 10 wells of a microtitre plate equals the analysis of 10×10^5 cells), which leads to highly reproducible results.

Furthermore, differences in the colour intensity of the immunocytochemically stained cells are not quantifiable by microscopic evaluation. We observed marked variation in this respect between patient samples and controls, which may reflect up-regulation of the number of binding sites expressed per cell (1).

Finally, the immunoenzymometric assay provides a setting suitable for studying modulatory effects of cytokines or other agents on the expression of ICAM-1 on the surface of alveolar macrophages in vitro. In summary we see this method as an approach to a better semi-quantitative identification of adhesion molecules on the surface of alveolar macrophages, compared with conventional immunocytochemical staining.

The main finding of the clinical part of this study was that ICAM-1 expression on the surface of alveolar macrophages is differently regulated in patients with sarcoidosis (mononuclear cell alveolitis) and bacterial pneumonia (neutrophil alveolitis). Sarcoid alveolar macrophages expressed significantly higher amounts of ICAM-1 than macrophages of healthy volunteers. Our data are consistent with recent work demonstrating increased expression of ICAM-1 on the surface of alveolar macrophages from patients with pulmonary sarcoidosis using immunocytochemical staining (8, 9). We further demonstrated that ICAM-1 expression of control alveolar macrophages can be significantly increased by stimulation with either TNF-α or interferon-γ. It has been demonstrated previously that ICAM-1 expression can be induced by cytokines on various cell types (1), but no data were hitherto available on the in vitro induction of ICAM-1 on the surface of alveolar macrophages. In contrast, ICAM-1 expression on the surface of alveolar macrophages from patients with bacterial pneumonia was not elevated, proving that upregulated ICAM-1 expression is not an unspecific finding in pulmonary inflammation. In the light of present knowledge, ICAM-1 expression on the alveolar macrophages surface seems to be crucial in the pathogenesis of lymphocyte accumulation and activation in the pulmonary compartment (10, 29). In neutrophil alveolitis different mechanisms of bronchoalveolar cell activation are likely to be involved.

Since alveolar macrophages can be harvested in large numbers from sites of disease activity by bronchoalveolar lavage, rapid and reliable identification of adhesion molecules such as ICAM-1 on the surface of cells from the pulmonary compartment might be a useful tool for further elucidation of the pathogenesis of these diseases.

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